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MORAXELLA BOVIS CYTOTOXIN AND CELL DETACHMENT FACTOR (SERINE PROTEASE, INFECTIOUS BOVINE KERATOCONJUNCTIVITIS)
Author: HALENDA, RUTH MARRION
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Supervisor: LELA K. RILEY
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of matrix. Histologically, the elastic media is degraded and a large inflammatory infiltrate is present. The present study characterises the changes in matrix proteins and the elastin degrading matrix metalloproteinases MMP-2 and MMP-9 in AAA compared to non-dilated, atherosclerotic occlusive disease tissue (AOD), and nondiseased aorta. Additionally, as invading inflammatory cells can release cytokines which mediate changes in matrix and MMP expression, representative cytokines were assessed to determine cytokine expression patterns in AOD and AAA.

There was a circumferential absolute increase in elastin, collagen, and total protein in AAA compared to AOD and non-diseased aorta. These data demonstrate that matrix synthesis is ongoing in AAA. However, the rate of increase in circumferential elastin content was less than the rate of increase in total protein and collagen. This results in progressive relative depletion of elastin with increasing AAA size. Additionally, elastin may be lost via degradation by MMPs. MMP-2 was increased in AAA compared to AOD at both the RNA and protein level. The activation state of MMP-2 was also increased in aneurysmal tissue in tight association with the matrix, suggesting that AAA tissue has a greater ability to activate proMMP-2. As MMP-2 is a collagenase as well as an elastase, it may play a pivotal role in degradation and remodeling in AAA. By ELISA, the pro-inflammatory cytokines IL-6 and TNF-α were increased in AOD, while IL-10 expression was higher in AAA. These unique cytokine patterns may explain the differences in matrix and MMP expression between AOD and AAA tissue. These findings suggest that rather than a simple degenerative process, aneurysm formation is a complex process of synthesis and degradation resulting in matrix remodeling and vessel wall growth.

Mechanisms of tissue destruction in inflammatory eye diseases. DiGirolamo, Nick, Ph.D. *University of New South Wales (Australia)*, 1998. 1pp.

Inflammatory eye diseases (IEDs) such as necrotising scleritis and anterior uveitis (AU), are sight-threatening conditions of the anterior portion of the eye. The pathogenesis and mechanism(s) of disease progression of these conditions are not well understood. Such diseases are characterised by extensive infiltration of inflammatory cells and remodelling of the extracellular matrix which forms the scaffold of the eye. The matrix metalloproteinases (MMPs) are a class of proteolytic ensymes active against connective tissue proteins. These ensymes are constitutively expressed by both resident and inflammatory cells, regulated by pro-inflammatory cytokines, and inhibited by tissue inhibitors of MMPs (TIMPs). The major aim of this study was to elucidate the potential role(s) of MMPs in IEDs and characterise their cellular sources. The expression of MMPs and TIMP-1 and their cellular sources was examined on archival tissue from patients with scleritis by *in situ* hybridisation and immunohistochemistry. Gelatinase B and stromelysin-1 mRNAs were localised to resident human scleral fibroblasts (HSF), macrophages, T-lymphocytes, and plasma cells, whereas TIMP-1 mRNA was less abundant. In addition, the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) was found in abundance in plasma cells in scleritis tissue. Analysis of total RNA and conditioned media from cultured HSF and a plasma cell line demonstrated increased expression of gelatinase B and stromelysin-1 when compared with TIMP-1. Zymography and immunoblotting of aqueous humor (AH) samples derived from patients with AU, revealed that gelatinase B and stromelysin-1 were over-expressed in contrast to TIMP-1. Similarly, increased levels of gelatinase B were found in AH derived from rabbits with endotoxin-induced uveitis (EIU). The results of this study provide strong *in vivo* and *in vitro* evidence for the hypothesis that an imbalance between ensyme/inhibitor ratio may be an underlying mechanism of tissue destruction characteristic of IEDs. The results also highlight the importance of plasma cells in an inflammatory response, not simply as antibody producers, but as potent secretors of TNF-α and MMPs, which are both mediators of tissue destruction and remodelling.

Mast cells and neutral proteinase in rheumatoid arthritis. Gotis-Graham, Ian, Ph.D. *University of New South Wales (Australia)*, 1997. 1pp.

The role of mast cells in rheumatoid arthritis (RA) remains uncertain, The aims of this thesis were to define synovial mast cell responses in early and advanced RA; to determine whether tryptase and stromelysin colocalise; to examine for correlations between chymase and a potential inhibitor, thrombospondin 1 (TSP1). Double immunohistochemical staining was used to distinguish mast cells as tryptase only (MC_T), tryptase/chymase (MC_{TC}), or chymase only positive (MC_C). The mean mast cell density was significantly increased in both early (40.3 cells/mm^2) and advanced RA (60.9 cells/mm^2), compared to osteoarthritic (21.7 cells/mm^2) or normal synovia (9.4 cells/mm^2). In early RA, MC_T were predominant, were found close to inflammatory cells and their density correlated with the inflammation index ($r = 0.4$, $P < 0.05$). Longitudinal studies showed that mast cell density and the predominant subset varied. In advanced RA, MC_{TC} expanded in areas of cellular connective tissue, especially in patients with more severe disease. MC_C cells were not identified. Using double immunohistochemistry, tryptase and stromelysin often colocalised in areas of intense inflammatory activity in RA synovium. The presence of stromelysin correlated with the thickness of the lining layer ($r = 0.85$, $P < 0.05$). Immunohistochemistry was used to localise TSP1 in synovial tissue. TSP1, especially the $TSP^{0.1}$. isoform, was highly upregulated in RA compared to the other groups and correlated with elastase and cathepsin G postive cells. Despite the structural similarities between cathepsin G and chymase, there was no correlation between TSP1 and chymase expression. Taken together, these results implicate the MC_T subset in the active inflammatory process, and the MC_{TC} subset in the destructive or reparative process of RA. Longitudinal studies confirmed the dynamic nature of the mast cell response. Tryptase may be important in activating stromelysin in areas of intense inflammatory activity. $TSP1^{0.1}$, with the most potent inhibitory activity *in vitro*, was specifically upregulated in RA. in proportion to the numbers of elastase/cathepsin G positive leukocytes. One role of TSP1 may be to act as a matrix-based regulator of these proteinases. These results provide new information about the roles of mast cells, leukocyte proteineses, TSP1 and stromelysin in rheumatoid synovitis.

***Morazella bovis*, cytotoxin and cell detachment factor.** Halenda, Ruth Marrion, Ph.D. *University of Missouri - Columbia*, 1998. 78pp. Supervisor: Lela K. Riley **Order Number DA9901239**

Morazella bovis, a gram-negative bacterium, is the causative agent of infectious bovine keratoconjunctivitis (IBK). This bacterium is unique in its ability to infect the bovine cornea in the presence of an intact epithelium. The purpose of this work was to identify virulence factors which may enable the organism to penetrate corneal epithelium.

A transformed corneal epithelial cell line was developed so that we would have a uniform population of cells with which to perform experiments. The cells were incubated with *Morazella bovis* culture filtrate, which was prepared by growing bacteria in broth and filtering out whole organisms. Incubation with bacterial culture filtrate resulted in detachment of cells from the substrate. Lack of uptake with trypan blue dye indicated that cells were alive, and the effect of filtrate was reversible. Characterisation of this activity, which we termed cell detachment factor, indicates that it may be a protein with serine protease activity.

The effect of live *M. bovis* organisms on cultured corneal epithelial cells was studied, and the bacteria were found to be cytotoxic. The expression of cytotoxin, CDF, and two other previously described virulence factors, hemolysin and leukocidin, were examined in a number of strains of *M. bovis*. Cytotoxin, hemolysin and leukocidin were produced only by pathogenic strains, while CDF was produced by all strains. Different mechanisms of action of cytotoxin and hemolysin are suggested by the observation that osmotic protectants prevent hemolysis by *M. bovis* but do not prevent lysis of corneal epithelial cells. A model for the interaction of CDF and cytotoxin in IBK is proposed in the Summary of this dissertation.

Cytophotometric analysis of neuronal RNA and protein in relation to beta-amyloid protein in the hippocampal formation, basal forebrain, cingulate gyrus, and visual cortex of patients with Alzheimer's disease. Kan, Robert Kwai-Hong, Ph.D. *The Pennsylvania State University*, 1998. 259pp. Adviser: Adam Anthony **Order Number DA9901046**

The present study was designed to examine Alzheimer's disease (AD)-induced changes in total neuronal RNA and protein content in relation to β-amyloid protein, and the extent of neurofibrillary tangles, neuritic plaque, β-amyloid plaque in the hippocampal formation, basal forebrain, cingulate gyrus, and visual cortex. Brain regions were

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Immunogenicity of Moraxella bovis hemolysin .
Ostle A G; Rosenbusch R F
American journal of veterinary research (UNITED STATES) May 1985, 46 (5) p1011-4, ISSN 0002-9645 Journal Code: 0375011
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Moraxella bovis hemolysin .
Ostle A G; Rosenbusch R F
American journal of veterinary research (UNITED STATES) Sep 1984, 45 (9) p1848-51, ISSN 0002-9645 Journal Code: 0375011
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Moraxella bovis hemolysin

A. G. Ostle, PhD, and R. F. Rosenbusch, DVM, PhD

SUMMARY

Moraxella bovis hemolysin was readily filterable through polycarbonate membrane filters, but not through nitrocellulose filters. The hemolysin was filterable through polycarbonate filters with pore diameters of ≥ 0.015 μm (APD). Of the hemolytic activity of cell-free filtrates, 74% could be pelleted by ultracentrifugation at 100,000 × g for 2½ hours. Hemolytic activity could be demonstrated in preparations of outer membrane fragments isolated from log-phase cultures. Hemolysin in *M bovis* broth cultures reached a maximum concentration in late logarithmic phase (4.5 hours after inoculation) and declined thereafter. Hemolysin was inactivated by heat, trypsin, formalin, and lyophilization.

Moraxella bovis is associated with the occurrence of infectious bovine keratoconjunctivitis (IBK), in association with *Mycoplasma bovoculi* or predisposing factors such as exposure to UV light.[1-3] *Moraxella bovis* produces a β-hemolysin that is apparent on bovine blood agar and is associated with pathogenicity in IBK.[4,5] Despite the apparent importance of the hemolysin in IBK, the few studies[6,7] on the hemolysin itself are contradictory.

Moraxella bovis hemolysin has been described as being filterable through a 0.22-μm (APD) membrane filter and can be destroyed by heat, formalin, or trypsin.[6] Hemolysin production was associated with logarithmic phase growth, reaching a maximum in late log phase, and declining thereafter. *Moraxella bovis* hemolysin was not filterable through a 0.45-μm membrane filter and could not be separated from the bacterial cells by centrifugation.[7] This hemolysin was also destroyed by heat, formalin, and trypsin. Hemolysin is also activated by divalent cations such as calcium.[6,7] One report[4] indicated that the hemolysin was not filterable through a ceramic filter.[4]

The purpose of the present report was to investigate the properties of *M bovis* hemolysin with regard to its cellular location and stability.

Materials and Methods

Bacterial cultures—Moraxella bovis strain 118F, a field isolate from cattle with IBK, was used.[1] The identity of the isolate was confirmed by fluorescent antibody reaction and biochemical testing.[8] *Moraxella bovis* 118F is β-hemolytic, caseinase-positive, penicillin-resistant, and doxycycline-sensitive. Cultures were grown on 5% bovine blood agar plates,[a] harvested into gram-negative wash solution,[1] and held in 1-ml volumes at −67 C until use.[1]

Tryptose-bovine serum albumin broth was used as a growth medium for bacterial cultures.[b] *Moraxella bovis* 118F (200 ml) was grown in 500-ml Erlenmeyer flasks at 37 C with shaking.[c] A 1.0% inoculum of 24-hour old cells was used. Cultures were harvested at 4.5 hours after inoculation.

Hemolysin assay—The hemolytic activity of *M bovis* cultures and culture fractions was assayed by a modification of the method of Sandhu and White.[6] Briefly, the diluent buffer was modified by the addition of 0.01% sodium azide to retard bacterial respiration and growth during the assay. This concentration of sodium azide had no effect on the lysis of bovine RBC within 24 hours.

A 1.0% washed bovine RBC suspension in isotonic saline solution + 0.01*M* $CaCl_2$ was used as a standard reagent. The hemolytic culture filtrate was diluted sequentially from 1:2 to 1:1,024 in isotonic saline solution + 0.01*M* $CaCl_2$ and 0.02% sodium azide (1.5 ml final volume) in 12-well plastic test plates. A 1.5-ml quantity of standard bovine RBC suspension was added to each well. A well with 1.5 ml of diluent in place of sample was used as a control. Plates were incubated for 3 hours at 37 C. The solution was then removed, the remaining RBC were pelleted by low-speed centrifugation, and the optical density of the solution was determined at a wavelength of 540 nm.[d] Results were compared with a standard curve and the number of hemolytic units was determined.[6]

Sample preparation—Moraxella bovis 118F cultures were removed from a shaker at 4.5 hours, and the whole culture, filtrates, and supernatant fluids were assayed for hemolysin content.

Filtration was performed by passing the culture broth through polycarbonate membrane filters[e] (0.4, 0.2, 0.1, 0.08, 0.03, and 0.015 μm), nitrocellulose membrane filters[f] (0.45 and 0.22 μm), and ultrafiltration acrilic copolymer membrane filters[g] [300,000 molecular weight (mol wt) nominal cutoff]. Filtration was performed with ambient atmosphere and with nitrogen gassing to reduce the partial pressure of oxygen. The filtrate was monitored for sterility.

Low-speed centrifugation[h] to remove whole cells was performed on 5-ml quantities of culture broth at 2,000 × g for 10 minutes. Whole-cell preparations were washed 3 times in phosphate-buffered saline solution (PBSS, pH 7.4) and were

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[a] Tryptose blood agar base, Difco Corp, Detroit, Mich.
[b] 1% (w/v) Tryptose (Difco Corp, Detroit, Mich), 0.5% w/v NaCl (Fisher Scientific, Pittsburgh, Pa) 0.5% bovine serum albumin (Sigma Chemical Corp, St Louis, Mo).
[c] New Brunswick G-86 shaking waterbath at 175 rpm. New Brunswick Scientific Inc, New Brunswick, NJ.
[d] Gilford 2400 spectrophotometer, Gilford Instruments Laboratory Inc, Oberlin, Ohio.
[e] Nucleopore Corp, Pleasanton, Calif.
[f] Millipore Corp, Bedford, Mass.
[g] Amicon Corp, Lexington, Mass.
[h] General Laboratory Centrifuge, Ivan Sorvall Inc, Norwalk, Conn.

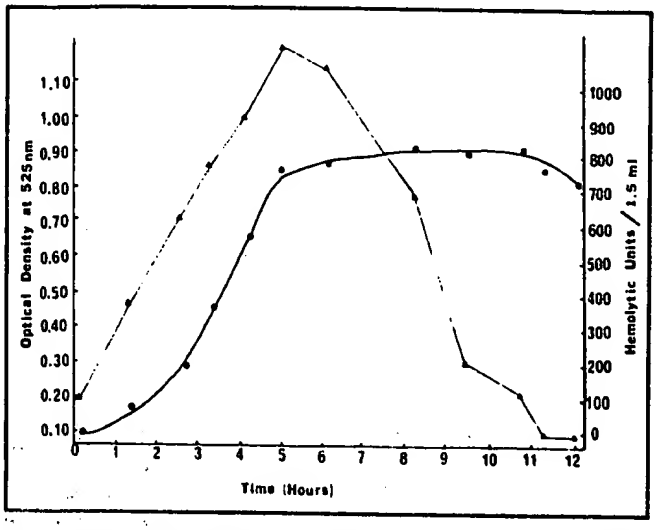

Fig 1—Growth and hemolysin production by *M bovis* 118F. Cells were grown in tryptose-BSA broth at 37 C with shaking. ● = Cell growth; ▲ = hemolysin activity in filtrates (0.4-μm polycarbonate filter).

TABLE 1—Hemolytic activity of *M bovis* broth culture fractions

Filtrate	Culture	Filtrate
	6,656	1,077
0.4-μm Polycarbonate	(100)	(16.2)
	6,878	955
0.1-μm Polycarbonate	(100)	(13.8)
	7,050	885
0.015-μm Polycarbonate	(100)	(12.5)
	8,112	0
0.45-μm Cellulose nitrate	(100)	(0)
	8,112	0
0.22-μm Cellulose nitrate	(100)	(0)
	8,512	0
300,000 mol wt Ultrafilter	(100)	(0)

Hemolytic activity expressed as hemolysin units/1.5 ml of sample. (% total activity for each treatment).

TABLE 2—Hemolytic activity of *M bovis* broth culture supernatants

Treatment	Culture	Filtrate	Supernatant	Pellet
Centrifugation				
2,000 × g	6,656	1,077	270	794
10 min		(100)	(25)	(74)
Centrifugation				
100,000 × g	4,486	1,112	0	822
2.5 hr	(100)	(25)	(0)	(18)
Outer membrane				
fragments*	5,733	993	0	769
	(100)	(17)	(0)	(13)

* Isolated outer membrane fragments released during cell growth.[9] Outer membrane fragments are represented by the pellet fraction.

Hemolytic activity expressed as hemolysin units/1.5 ml of sample (% of total activity for each treatment).

suspended in 5 ml of PBSS before use. High-speed centrifugation[1] was performed on 30-ml samples of culture broth filtered through 0.4-μm polycarbonate filters. These samples were centrifuged at 100,000 × g for 2.5 hours at 4 C.

Outer membrane fragments were isolated.[9] Briefly, 20 g of *M bovis* 118F cells was suspended in 200 ml of PBSS (pH 7.4), and whole cells were removed by 3 centrifugations at 5,000 × g for 10 minutes and 1 at 12,000 × g for 15 minutes. Outer membrane fragments in the supernatant fluid were pelleted by centrifugation at 100,000 × g at 4 C for 3 hours. The pelleted material was suspended in 3 ml of tris buffer (pH 7.4) and was stored at −67 C until use.

Hemolysin stability—The effect of trypsin, formalin, and L-cysteine on hemolysin stability was tested.[7] Trypsin was added to culture filtrate (0.4-μm polycarbonate) to a final concentration of 0.25%, and the solution was incubated at 37 C for 1 hour. Formalin was added to the culture filtrate to a final concentration of 0.1%, and the filtrate was incubated at 37 C for 15 minutes. L-Cysteine was added to the culture filtrate to a final concentration of 0.01*M* and the filtrate was incubated at 37 C for 30 minutes. Nontreated filtrate was incubated at the same times and temperatures used previously and was used as a control.

Various stabilizers and protease inhibitors were added to culture filtrates to determine their ability to prevent loss of hemolysin activity. Trypsin soybean inhibitor,[8] 2 mg/ml; dithiothreotol, 10^{-4}*M*; and diethylpyrocarbonate, 10^{-4}*M* were added to hemolysin-containing culture filtrates. Equal concentrations of these agents were added to 1% washed bovine RBC as a control. These filtrate preparations were stored at 4 C overnight and assayed for hemolytic activity.

Culture filtrates were quick frozen with dry ice-acetone baths and held at −67 C for 24 hours before to hemolysin assay. Quick-frozen filtrates were also lyophilized, hydrated in isotonic saline solution, and assayed for hemolysin.

To determine the effect of heat on hemolysin activity, culture filtrates were held at 56 C for 1 hour and assayed for hemolytic activity. Nontreated filtrates were used as a control.

Growth curves—Moraxella bovis 118F was grown in 200 ml of tryptose/bovine serum albumin (BSA) broth at 37 C with shaking as previously described. A 1% inoculum of 24-hour-old cells grown in an identical manner was used. Samples were removed at 30- or 60-minute intervals (depending on the stage of growth), and the optical density of each sample was determined at a wavelength of 520 nm.[4] Samples were also diluted in gram-negative wash and plated on bovine blood agar. Viable cell counts were made from these plates after incubation at 37 C for 24 hours. Samples were also assayed for hemolysin activity in whole cell broth and culture filtrate from 0.4-μm polycarbonate filters as previously described.

Polyacrylamide gel electrophoresis (PAGE)—Sodium dodecyl sulfate (SDS)-PAGE analysis of released outer membrane fragments and of outer membrane fractions isolated from whole cells was

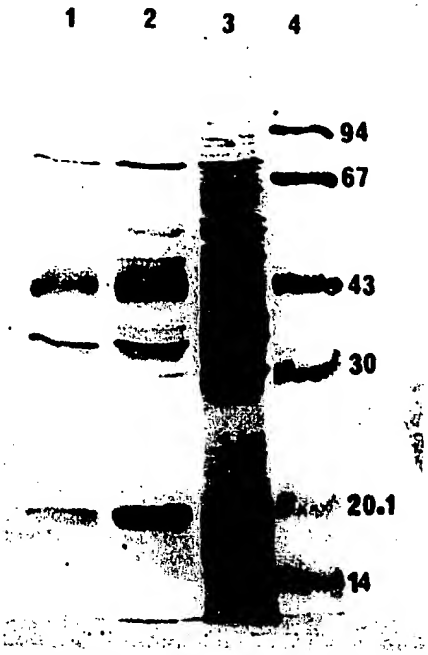

Fig 2—The SDS-PAGE of isolated outer membrane of *M bovis* 118F. Lanes: 1 and 2 - Purified outer membrane fragments released during growth of *M bovis* 118F; Lane 3 - Outer membrane obtained from whole, log-phase *M bovis* 118F; Lane 4 - mol wt × 10^3.

[1] L2-65B ultracentrifuge, 60 Ti rotor, Beckman Instruments, St Louis, Mo.

[7] Grand Island Biological Corp, Grand Island, NY.

TABLE 3—Effect of stabilization and concentration treatments on *M bovis* hemolysin

Treatment	Hemolytic units* (× 10^2)
Calcium addition (0.01*M* $CaCl_2$)	10.1
Magnesium addition (0.01*M* $MgCl_2$)	8.86
Trypsin (0.25%)	0
Formalin (0.1%)	0
L-Cystine (0.01*M*)	4.3
Trypsin soybean inhibitor (2 mg/ml)	100.8
Ditiothreotol (10^{-4}*M*)	9.96
Diethylpyrocarbonate (10^{-4}*M*)	9.40
Quick freezing	0.12
Lyophilization	0.18
No treatment†	5.66

* Hemolytic units/1.5-ml sample of *M bovis* 118F cultures. † Culture filtrates prepared as described previously and assayed for hemolysin activity without $CaCl_2$ in the hemolysin assay diluent. All other treatments, except magnesium addition, used standard assay buffer.

performed on 10% SDS gels at 30 mA.[9] Cells were lysed with a French press,[9] and the outer membrane fractions were isoalted[10]

Results

Hemolysin was readily filterable through polycarbonate filters with an APD of as low as 0.015 μm. Hemolysin was not filterable through nitrocellulose membrane filters, even those with an APD of 0.45 μm. Hemolysin was also not filterable by ultrafiltration membranes with a retentivity of ≥ 300,000 mol wt (Table 1).

Hemolysin was present in washed whole cell preparations suspended in PBSS. An average of 41,000 U of hemolysin/1.5-ml sample of washed whole cell preparations was detected.

Observed hemolysin activity in filtrates generally paralleled cell growth during logarithmic phase. Hemolysin activity declined rapidly with the onset of stationary phase (5 to 6 hours after inoculation, Fig 1) Hemolysin activity in whole culture preparations (not filtered) peaked at the same growth time that hemolytic activity peaked in the filtrates.

Culture filtrates centrifuged at 100,000 × g for 2.5 hours had hemolytic activity in the pellet only. Hemolytic activity was also apparent in preparations of released membrane fragments, but not in the supernatant fluids (Table 2).

[9] Basic techniques and exercises in electrophoresis. Hoefer Scientific Instruments, San Francisco, Calif.

[10] American Instrument Co, Rockville, Md.

Preparations of outer membrane material and released outer membrane fragments had identical SDS-PAGE profiles, indicating that outer membrane fragments are released during growth (Fig 2). Outer membrane preparations isolated from whole cells had hemolytic activity (Table 2).

Three major exterior proteins were detected on SDS-PAGE preparations of outer membranes isolated from whole cells. These protein bands correspond to identical bands on SDS-PAGE preparations of released membrane fragments.

Stability and activity of hemolysin—Additions of calcium or magnesium ions resulted in increased hemolytic activity in culture filtrates. Hemolytic activity was not observed after treatment with formalin, trypsin, or heat. Stabilizers, such as cysteine or dihydrothreotol, did not result in retention of hemolysin activity after filtrates were stored at 4 C overnight. Similarly, protease inhibitors diethylpyrocarbonate or trypsin soybean inhibitor did not prevent loss of hemolysin activity after overnight storage of the filtrate (Table 3).

Effect of sodium azide on hemolysin assay—Sodium azide added to the hemolysin assay buffer resulted in hemolysin titers approximately one-half those noticed when sodium azide was not included. An increase in bacterial numbers could be noticed on microscopic observation of assay wells at 4 hours of incubation when whole cell broth was assayed for hemolysin activity without sodium azide in the diluent. Viable *M bovis* could also be recovered after 24-hour incubation of these assay plates at 37 C, but not when sodium azide was added to the diluent. Similar results were obtained when sodium azide was replaced with doxycycline. Viable *M bovis* cells could not be recovered after the assay was complete when doxycycline was added to the assay diluent. Neither sodium azide nor doxyclycline lysed bovine RBC when added to diluent without hemolysin preparations.

Discussion

Moraxella bovis 118F produced a hemolysin in broth and solid media which had similar characteristics to hemolysins described previously.[6,7] These characteristics included inactivation by heat, formalin, and trypsin and activation by divalent cations such as magnesium and calcium.

The hemolysin was filterable through polycarbonate filters, but not through nitrocellulose filters. This is in contrast to the data of Nakazawa and Nemoto,[7] who indicated that the hemolysin was not separable from the cell body. However, these research workers used nitrocellulose filters, through which hemolysin is not readily filtered. A hemolysin from *M bovis* was filterable through nitrocellulose filters at up to approximately 50% efficiency.[6] We have been unable to demonstrate hemolysin activity in filtrates passed through nitrocellulose filters. The hemolytic activity that could be detected in broths passed through a polycarbonate filter was only about 16% of that present in the initial culture.

Hemolysin was produced during the logarithmic phase of cell growth.[6,7] However, hemolysin activity declined during stationary phase. This is in contrast to the data reported by Nakazawa and Nemoto,[7] who were able to maintain henolysin concentrations in their cultures for 120 hours. Because Nakazawa and Nemoto[7] were unable to produce culture filtrates with marked hemolytic activity, their hemolysin titers are based on tests of whole, viable cells mixed with 1% RBC at 37 C. This procedure might well result in further growth of the organism and/or hemolysin production. For this reason, sodium azide was added to the hemolysin assay diluent in our experiments, and a lower hemolysin activity was noticed after 4 hours of incubation in diluent with azide than in diluent without azide. Viable cells could also be recovered from hemolysin assay wells, using whole cell broth, when azide was not included in the diluent, but not when azide was present. Similar results were obtained when sodium azide was replaced by doxycycline. For this reason, it is likely that additional hemolysin is produced during the assay of viable cells unless steps are taken to limit such an occurrence.

Seemingly, the hemolytic activity of *M bovis* is filterable (under appropriate conditions) and capable of

being pelleted by high speed centrifugation. Hemolysin may be membrane-associated and may be present on membrane blebs or vesicles. The possibility of a membrane vesicle-associated hemolysin is supported by the presence of hemolytic activity in isolated membrane bleb fractions from broth cultures and on outer membrane fractions isolated from mechanically broken cells.

The presence of such enzymes on membrane blebs occurs on several occasions for toxins derived from the envelopes of various gram-negative bacteria.[11] These envelope-associated toxins occur in culture filtrates as a result of cell growth, not cell lysis. This is consistant with the release of filterable hemolysin in *M bovis* during logarithmic-phase growth and not during stationary phase. A membrane bleb location for the hemolysin of *M bovis* may be indicated because membrane fractions from young logarithmic-phase cultures were isolated that match the SDS-PAGE profiles of membranes isolated by mechanical breakage of whole cells. Both of these fractions contain hemolytic activity.

The stability of *M bovis* hemolysin is a distinct problem. Filtrates lost all or most of their hemolytic activity when quick frozen, lyophilized, or held overnight at 4 C. Stabilizers or protease-inhibitors used in this study were not able to cause a retention of hemolysin activity. When the poor stability of the hemolysin is considered together with the loss of hemolytic activity observed during stationary phase in broth culture, it is possible that antisera produced against 24-hour old cultures may not be optimal in terms of antihemolytic activity. Inoculation of animals with *M bovis* is performed frequently with stationary-phase cultures or cultures in which the precise stage of growth was not known.[12-14] If antihemolytic activity is a desired attribute of immunization programs with *M bovis* bacterins, use of late log-phase cultures would be preferable. Because hemolysin seems to be associated with the production of keratitis by *M bovis*, an antihemolytic factor may prove to be important in the protection of cattle against IBK.

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Production and Characterization of *Moraxella bovis* Hemolysin

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SUMMARY

Moraxella bovis hemolysin was produced in trypticase soy broth and maximum hemolytic activity of the culture was observed during the logarithmic phase of growth. The hemolysin was filterable through a 0.22-μm (APD) membrane filter, heat labile, and destroyed by treatment with formalin or trypsin. There was no difference in the amount of hemolysin production by rough or smooth colony types of an isolate, although differences were observed between 2 different isolates. Partial requirement of a sulfhydryl group and divalent cations were suggestive of an enzymatic nature of *M bovis* hemolysin.

Although *Moraxella bovis* is considered to be the etiologic agent of infectious bovine keratoconjunctivitis,[1,9,12,a] the mechanism by which this organism produces disease is not understood. Some investigators have suggested the involvement of potent toxins which are elaborated during growth.[5,6,11,a]

Beta hemolysis produced by *M bovis* cultures on blood agar plates was observed long before the hemolytic activity was demonstrated in embryonating chicken egg cultures.[5,8] The hemolysin was reported to be heat labile and inseparable from viable bacterial cells, and loss of hemolytic activity was observed by treatment with formalin or filtration through a Seitz EK pad.[5]

The purpose in the present investigation was to study and characterize *M bovis* hemolysin.

Materials and Methods

Isolates—Two isolates (both rough and smooth colony morphotypes) of *M bovis* IBH 68-712L[b] and FLA-264, were used for the study of hemolysin production.

Hemolysin Assay—The activity of hemolysin was determined by its lytic action on a 1% sheep erythrocyte suspension in saline solution containing 0.01 *M* CaCl. The assay was made by preparing twofold dilutions of hemolysin ranging from 1:2 to 1:1,024. An equal amount of erythrocyte suspension was added to 1.5 ml of each dilution. The tubes were incubated for 4 hours at 37 C followed by 12 to 18 hours at 4 C. After centrifugation at 1,500 × g to remove remaining erythrocytes, the optical density (OD) of the supernatant fluid of each dilution was recorded at 540 nm on a spectrophotometer.[c]

To standardize the assay procedure, 100%, 75%, 50%, 25%, and 12.5% hemolysis of a 1% erythrocyte suspension was produced with distilled water, keeping the total volume at 4 ml in all cases. These tubes were incubated and their optical densities were recorded in the same manner as in the experimental trials. A standard curve was constructed from an average of 10 different trials by plotting percentage hemolysis against OD readings. One hemolytic unit was defined as that amount of hemolysin which would lyse 1% of the erythrocytes in a standard suspension. The units in a particular sample were calculated by multiplying the reciprocal of hemolysin dilution by the hemolytic units obtained from the standard curve on the basis of the OD of that dilution.

Growth and Production of Hemolysin—The inoculum was prepared by selecting pure rough or smooth colonies from blood agar plates and inoculating 10 ml of trypticase soy broth.[d] The tubes were incubated at 37 C for 48 hours after which they were kept at 4 C. Before inoculation, the culture was shaken, and 2 ml was used to inoculate 100 ml of broth in a nephelometer flask. Duplicate flasks were used for each type. An uninoculated flask served as control. All flasks were incubated on a gyrotory incubator shaker[e] at 140 rpm at 37 C.

After the zero reading, OD readings at 525 nm were taken every 2 hours for the growth curve, and a sample of 4 ml was withdrawn aseptically. A 1.5-ml portion was used to assay hemolysin in whole culture, and the remainder was filtered through a sterile 0.22-μm (APD) filter.[f] Heavy cultures were centrifuged at 3,000 × g to remove most of the cells prior to filtration. Hemolysin assay was run on 1.5 ml of the filtered culture medium.

Cell-free culture filtrate was used in all further experiments, since there was complete loss of hemolytic activity when routine procedures for concentration and purification (ammonium sulfate precipitation, acid precipitation, or cold methanol precipitation) were used.

Hemolytic Activity of M bovis Hemolysin on Erythrocytes of Various Species of Animals—The activity of *M bovis* hemolysin was determined on erythrocytes from the cow, rabbit, horse, and man, in addition to erythrocytes from sheep.

Effects of Calcium and Magnesium Ions on Hemolytic Activity—This was determined by addition of 0.01 *M* $CaCl_2$ or $MgCl_2$ to physiologic saline solution for dilution of hemolysin and for suspension of sheep erythrocytes.

Effects of Trypsin, Heat, and For-

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[a] Pugh, G. W., Jr.: *Moraxella bovis* and Its Relationship to Bovine Infectious Keratoconjunctivitis. PhD dissertation submitted to the graduate faculty, Iowa State University, Ames, Ia, 1969.

[b] Obtained from Drs. D. E. Hughes and G. W. Pugh, Jr., National Animal Disease Center, Ames, Ia.

[c] Spectronic 20, Bausch & Lomb, Inc. Analytical Systems, Ophthalmic Division, Scientific Optical Products Division, Rochester, NY.

[d] BBL, BioQuest Division, Becton, Dickinson and Company, Cockeysville, Md.

[e] New Brunswick Scientific Company, Inc, New Brunswick, NJ.

[f] Millipore filter, Millipore Filter Corporation, Bedford, Ma.

malin Treatment on Hemolysin—A 2% solution (w/v) of trypsin 1:250,[a] prepared in phosphate-buffered saline solution (pH 7.6), was added to hemolysin to give a final trypsin concentration of 0.25%. The mixture was incubated in a water bath at 38 C. Samples were taken at 0, 15, 30, and 60 minutes and assayed for hemolytic activity. Hemolysin, diluted in the same manner but without trypsin treatment, was included as a control.

To determine the effect of heat, the hemolysin was subjected to 56 C for 1 hour in a water bath, while the effect of formalin treatment was determined by the addition of formol to obtain a final concentration of 1% (v/v) and allowing the mixture to stand at 4 C for 1 hour.

Effect of Iodoacetic Acid and Cysteine on Hemolytic Activity—To demonstrate if the activity of *M bovis* hemolysin was dependent on —SH groups, as in the case of some enzymes, the effects of iodoacetic acid and cysteine were determined. Bacterial culture was centrifuged at 13,820 × g for 2 hours to remove the cells, since filtration resulted in a decrease of hemolytic activity. The hemolytic activity of hemolysin used in the present experiment was about twice that of others due to unknown reasons. Equal amounts of 0.02 *M* iodoacetic acid and supernatant culture fluid were mixed and left at room temperature for 30 minutes, after which the culture was assayed for hemolytic activity. The solution of cysteine hydrochloride was prepared similarly, except that it was brought to a neutral pH before use, and it was allowed to react with hemolysin for 10 minutes before assaying. Iodoacetic acid and cysteine solution controls were included in the experiment.

Results

Activity of Hemolysin—Hemolytic activity of rough and smooth morphotypes of *M bovis* isolate FLA-264 is shown (Fig 1). Filterable hemolysin paralleled that of hemolysin in whole culture, with peak activity at 10 to 12 hours after inoculation. Irrespective of colony type of the isolate, hemolytic activity was observed in the logarithmic phase of growth. Isolate IBH68-712L hemolysin followed a similar kinetic pattern. Although there was no difference in the hemolytic activities of rough and smooth morphotypes of isolates, significant quantitative differences were observed in the hemolytic activity of 2 different isolates, FLA-264 and IBH68-712L (Fig 1 and 2).

[a] Difco Laboratories, Detroit, Mi.

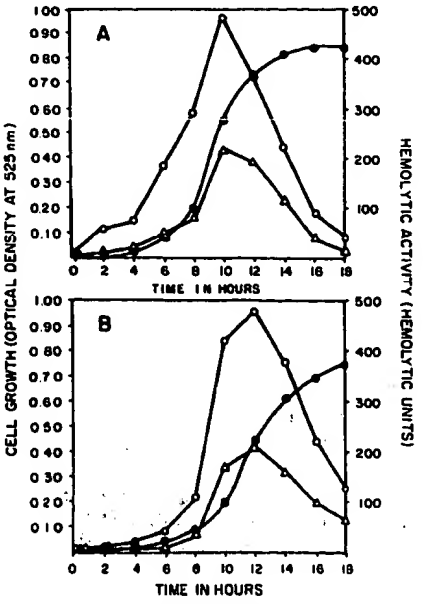

Fig 1—Hemolysin production by *Moraxella bovis* isolate FLA-264; A, rough morphotype; B, smooth morphotype. Cells were grown in trypticase soy broth, and hemolysin was assayed against sheep erythrocytes. ●—●, cell growth; ○—○, hemolysin in whole cultures; △—△, filterable hemolysin.

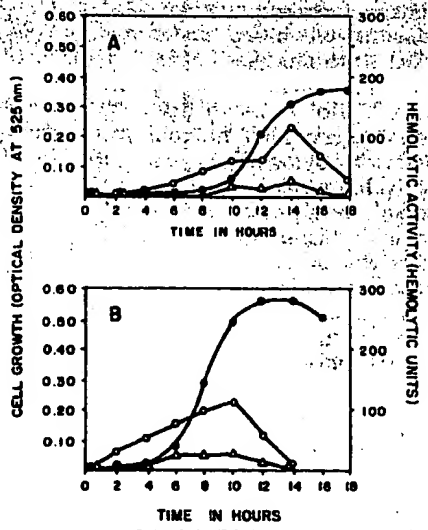

Fig 2—Hemolysin production of *M bovis* isolate IBH68-712L; A, rough morphotype; B, smooth morphotype. Cells were grown in trypticase soy broth, and hemolysin was assayed against sheep erythrocytes. ●—●, cell growth; ○—○, hemolysin in whole culture; △—△, filterable hemolysin.

Hemolytic Activity of Erythrocytes of Various Species of Animals—Moraxella bovis hemolysin lysed erythrocytes from rabbit, sheep, cow, horse, and human blood (Table 1). Rabbit erythrocytes were most susceptible, with sheep, cow, and horse erythrocytes in order of decreasing susceptibility; erythrocytes from man were least susceptible.

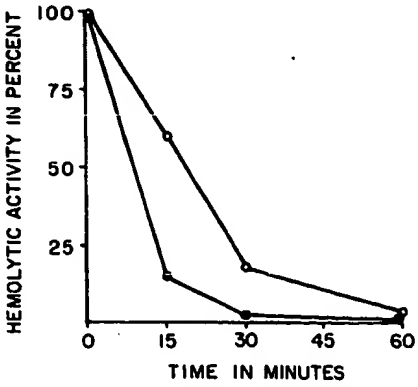

Fig 3—Effect of 0.25% trypsin on *M bovis* hemolysin. ●—●, trypsin treated; ○—○, no treatment (control).

TABLE 1—Hemolytic Activity of *Moraxella bovis* Hemolysin on the Erythrocytes of Various Species of Animals

Source of erythrocytes	Hemolytic activity (hemolytic units (HU))
Rabbit	332
Sheep	215
Cow	175
Horse	140
Man	48

TABLE 2—Effects of Calcium and Magnesium Ions on the Activity of *M bovis* Hemolysin

Cation	Hemolytic activity (HU)
None	98
Ca^{2+} (0.01 *M* $CaCl_2$)	183
Mg^{2+} (0.01 *M* $MgCl_2$)	108

TABLE 3—Effects of Iodoacetic Acid and Cysteine on the Hemolytic Activity of *M bovis* Hemolysin

Source of hemolysin	Hemolytic activity (HU) after treatment with— Iodoacetic acid	Cysteine	None (control)
FLA-264-rough	470	390	900
FLA-264-smooth	340	235	960

Effects of Calcium and Magnesium Ions on Hemolytic Activity—The addition of calcium or magnesium ions enhanced the activity of *M bovis* hemolysin (Table 2).

While in the presence of Mg^{2+}, there was a slight increase in the hemolytic activity, it almost doubled after addition of Ca^{2+}.

Effects of Trypsin, Heat, and Formalin Treatment on Hemolytic Activity—The treatment of *M bovis* hemolysin with 0.25% trypsin resulted in loss of 85% of hemolytic activity in 15 minutes (Fig 3). Some loss of activity was also observed by incubation of hemolysin without trypsin at 38 C. When treated with formalin or heat at 56 C for 1 hour, hemolytic activity was lost.

Effects of Iodoacetic Acid and Cysteine on Hemolytic Activity—There was partial inactivation of the hemolysin by treatment with iodoacetic acid (Table 3). One sample lost 48%, whereas the other sample lost 65% of hemolytic activity. Cysteine treatment did not result in reactivation of hemolytic activity of *M bovis* hemolysin, which had been lost by ammonium sulfate precipitation. In contrast, hemolytic activity of the culture supernatant was decreased after cysteine treatment.

Discussion

Results of the present investigation showed that *M bovis* produced hemolysin in artificial medium (trypticase soy broth). Although *M bovis* hemolysin was demonstrated in embryonating chicken egg cultures,[3] the hemolytic activity was lost when cultures were filtered through a Seitz EK pad. The present study showed that the hemolysin in culture could be filtered through a 0.22-μm membrane filter, but with some decrease in activity.

The parallel correlation of hemolysin activity of whole culture and that of culture filtrate indicated that probably there was only 1 type of hemolysin produced by *M bovis*. The hemolysin molecules were perhaps closely associated with the cell surface before their release into the culture medium. The hemolysin was produced during the logarithmic phase of the growth cycle with its peak in the last half of the logarithmic phase. There was no difference in the amount of hemolytic activity produced by rough or smooth types of an isolate, although marked differences were observed between both types of 2 different isolates. Since it was reported that nonhemolytic *M bovis* isolates did not produce characteristic lesions in the eyes of cattle,[10] hemolysin production may be an important factor in differences of virulence among various isolates.

Biologically, *M bovis* hemolysin may be protein, since it was heat labile and could be destroyed by proteolytic enzyme. The enhancement in activity by addition of calcium or magnesium ions, along with the sigmoid pattern of curves obtained when hemolysin was plotted as a function of concentration, indicated the enzymatic nature of *M bovis* hemolysin.[3]

Partial loss in hemolytic activity by iodoacetic acid treatment demonstrated that the sulfhydryl group was partially required for hemolytic activity, as reported for *Listeria monocytogenes*,[7] but lack of activation by cysteine treatment was not clear. The explanation given by Fruton and Bergman,[4] that the reducing agents acted as coenzymes and formed a dissociable complex with only particular enzymes, may be a possibility. Another factor which may have caused instability of *M bovis* hemolysin in culture or cell-free filtrate was the production of extracellular proteolytic enzymes.[2]

Moraxella bovis hemolysin was very labile to routine procedures of concentration and purification. Studies in this direction are needed to relate hemolytic activity to pathogenicity of this organism.

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